

# Ultraviolet radiation-absorbing mycosporine-like amino acids (MAAs) are acquired from their diet by medaka fish (*Oryzias latipes*) but not by SKH-1 hairless mice

Donald S. Mason<sup>a</sup>, Freya Schafer<sup>a</sup>, J. Malcolm Shick<sup>a,\*</sup>, Walter C. Dunlap<sup>b</sup>

<sup>a</sup> Department of Biological Sciences, University of Maine, 5751 Murray Hall, Orono, ME 04469-5751, USA

<sup>b</sup> Australian Institute of Marine Science, PMB No. 3, Townsville MC, Qld. 4810, Australia

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## Abstract

To assess whether vertebrates can acquire, from their diet, ultraviolet radiation-absorbing mycosporine-like amino acids (MAAs), medaka fish and hairless mice were maintained for 150 and 130 days, respectively, on diets either including *Mastocarpus stellatus* (rich in MAAs) or the same diets without this red alga. In medaka, the MAAs palythine and asterina-330, present in trace quantities in the diet with added *M. stellatus*, were present in significantly greater quantities in the eyes of fish fed this diet than in the eyes of control fish. Only traces of MAAs were present in the skin of medaka fed the diet containing MAAs. Shinorine, the principal MAA in *M. stellatus*, was not found in any tissues of medaka, which raises questions about the specificity of transport of MAAs. In hairless mice, no dietary MAAs were found in the tissues of the eyes, skin, or liver after maintenance on the experimental diet. Low concentrations of shinorine were present only in the tissues of the small and large intestines. These results indicate that MAAs are acquired from their diet and translocated to superficial tissues by teleost fish, but that mammals may be incapable of such. Thus, dietary supplementation with MAAs may be useful in aquacultured species of fish, but MAAs as 'dietary sunscreens' may not be an option for mammals, including humans. Nevertheless, our demonstration of the uptake of shinorine by human skin cancer cells in culture raises evolutionary questions regarding the organ specificity of the capacity for the cellular transport of MAAs. © 1998 Elsevier Science Inc. All rights reserved.

**Keywords:** MAA uptake; Mycosporine-like amino acids; Ultraviolet radiation; Sunscreens

## 1. Introduction

Solar ultraviolet radiation (UVR) is one of the most abundant and common cancer-causing agents to which both aquatic and terrestrial animals are exposed. The deleterious effects of solar UVR have been extensively studied in animal models, including the medaka fish *Oryzias latipes* [18,19] and the SKH-1 hairless mouse *Mus musculus* [10]. Solar UVR is one of the etiological agents in, among many others, epidermal pathologies in fish [5,6,24], ocular damage in a variety of vertebrates

[44], as well as skin cancers and immune suppression in hairless mice [10,30].

UVR exerts its deleterious effects either by direct damage to proteins, DNA, and lipids, which absorb wavelengths within the range of environmentally relevant UVR (295–400 nm), or indirectly via generation of reactive oxygen species (ROS) [41]. Once UVR has reached and damaged its cellular targets, it may overwhelm the cell's ability to repair this damage, resulting in the aforementioned pathologies. Thus, it would be beneficial to intercept UVR before it reaches critical cellular targets. Consequently, animals have evolved mechanisms that block or attenuate UVR. One such mechanism, a suite of UVR-absorbing mycosporine-

\* Corresponding author. Tel.: +1 207 5812562; fax: +1 207 5812537; e-mail: shick@maine.edu

like amino acids (MAAs), putative UVR sunscreens, is found nearly ubiquitously among marine invertebrates [29,34], and in the lenses of freshwater [39] and marine teleost fishes [16,39]. The presence of MAAs in terrestrial animals has not been shown.

MAAs are a class of water-soluble compounds characterized by either a cyclohexenone or cyclohexenimine ring conjugated with the nitrogen substituent of an amino acid [15] (Fig. 1). MAAs are hypothesized to act as sunscreens by absorbing the energy of ultraviolet radiation before it reaches cellular targets and harmlessly dissipating this energy as heat. However, at least one MAA, mycosporine-glycine ( $\lambda_{\max} = 310$  nm), may also function as an antioxidant in marine organisms [17], particularly photoautotrophic symbioses [15].

The role of MAAs as natural sunscreens has been inferred largely from: their high molar extinction coefficients at wavelengths within the solar UVR spectrum (i.e. the 19 known MAAs have individual absorption maxima between 309 and 360 nm); the inverse relationship between depth (itself inversely related to fluence of UVR) and MAA concentration in corals [13,22,36]; and evidence that MAAs are often most concentrated in tissues exposed to UVR, e.g. the epidermis of coral reef holothuroids [35], the eggs of the broadcast-spawning temperate sea urchin *Strongylocentrotus droebachiensis* [7], and the lenses of freshwater [39] and marine teleosts [16,39].

The ring structure of MAAs is probably a product of the shikimic acid pathway, which is not found in metazoans but limited to bacteria, algae, fungi, and higher plants [4,43]. Thus in marine symbioses such as

zooxanthellate corals, MAAs are assumed to be synthesized by endosymbiotic algae and translocated to their invertebrate host [3,14], although this may not be the case in all invertebrate/algal symbioses [3,38]. The finding that taxonomically diverse metazoans not harboring endosymbiotic algae also have MAAs [8,16,23,29,35] suggests other origins of the compounds in such cases. The two main hypotheses for their acquisition are that MAAs are synthesized by the enteric bacterial flora and absorbed by the animal, and that MAAs are acquired from their diets by marine consumers.

The dietary acquisition of MAAs has been suggested by several investigators, most strongly in Ref. [35], where the acquisition of MAAs from their diet was hypothesized in coral reef holothuroids based on the presence of MAAs in the coral sand, including cyanobacteria and unicellular algae, on which the animals feed. The apparent removal of MAAs during passage through the digestive tract (Dunlap, Shick and Larsen, in preparation) further supports such a hypothesis. The dietary acquisition and accumulation of MAAs from a diet of the marine red alga *Mastocarpus stellatus*, rich principally in the MAA shinorine, was confirmed experimentally in the ovaries of the green sea urchin *Strongylocentrotus droebachiensis* maintained on a controlled diet of this alga [7]. Embryos of this sea urchin from adults fed an MAA-rich diet showed a significant, MAA concentration-dependent protection from UVR-induced cleavage delay compared with those from urchins fed a diet lacking MAAs [1].

These studies, and the observation that wild-caught fishes have MAAs in their eyes [16,39], prompted us to test whether two phylogenetically distant vertebrates used as models in cancer research, the medaka fish, *Oryzias latipes*, and the SKH-1 hairless mouse, *Mus musculus*, are capable of acquiring MAAs from a diet including *M. stellatus*. To accomplish this, we maintained medaka fish and hairless mice for  $\geq 130$  days on controlled diets formulated with or without *M. stellatus* (i.e. with or without MAAs) and analyzed their tissues for the appearance of the MAAs present in their diets. We also studied human skin cancer cells (A431) in culture to test whether they can accumulate MAAs from the medium. This cell line is derived from an epidermal basal cell carcinoma [21] and lacks the melanin and keratin that would complicate further studies of effects of UVR; the lack of UV-blocking melanin also renders basal cells susceptible to UVR. The logical extensions of our studies would be to test whether dietary fortification of vertebrate tissues with MAAs provides protection against UVR-induced effects and whether ingestible algal sunscreens may be an option in the future.

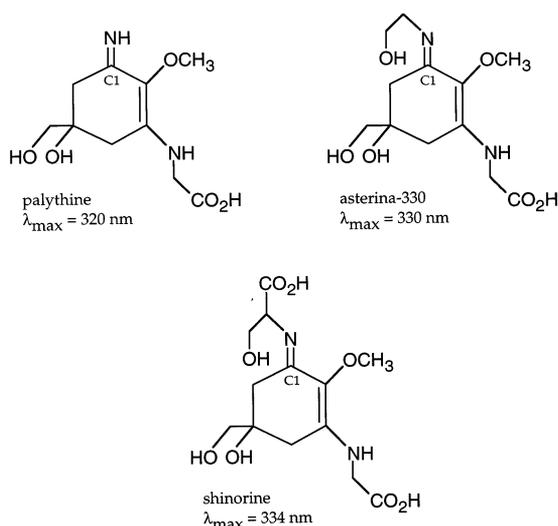


Fig. 1. Chemical structures of the mycosporine-like amino acids present in *Mastocarpus stellatus* used to formulate the experimental diets.

## 2. Materials and methods

### 2.1. Experimental mice

Five- to 6-week-old female SKH-1 hairless mice (Charles River Laboratories, Portage, MI) were used in this study. After arrival in our laboratory, mice were housed three to a cage and supplied with water and a standard laboratory diet (Agway Prolab 3000) ad libitum. The room was maintained at 22°C on a 12-h light/12-h dark cycle throughout the study. The room was monitored for ambient ultraviolet radiation with an International Light IL1400A radiometer and model SEL033 UVA and SEL240 UVB sensors (International Light, Newburyport, MA). There was no detectable UV emitted by the lights in the room.

Two weeks after arrival, the mice were randomly divided into two experimental groups: those fed the standard laboratory diet alone and those fed the standard laboratory diet with the addition of 5% (w/w) of a freeze-dried red alga, *Mastocarpus stellatus*. The cages were randomly rotated among spaces on the holding racks to control for the variability of visible light in the room. After the initial 2-week period on the standard laboratory diet, four mice were sacrificed and their component tissues were treated as described below for determination of initial MAA concentration. Thereafter, four mice from each experimental group were removed at 37, 80, and 130 days and their tissues analyzed for the presence of MAAs as described below.

### 2.2. Preparation of experimental diet for mice

*Mastocarpus stellatus* was collected from Schoodic Point, Maine, USA, in September 1996, cleaned of epibionts, and stored frozen at –20°C until ground with a mortar and pestle in liquid nitrogen, frozen at –80°C, and lyophilized. The dried alga was ground to an even powder in a Wiley mill fitted with a #40 mesh screen (Arthur H. Thomas Co., Philadelphia, PA). Ground *M. stellatus* was thoroughly mixed with ground Prolab 3000. Distilled water was added to form a slightly cohesive mixture and the diet was reformed into pellets, allowed to dry, and stored at –20°C until fed to the mice.

### 2.3. Preparation of mouse tissue samples

Mice were killed by cervical dislocation. Tissue samples were excised from the mice as described below and briefly rinsed with isotonic saline. Tissue samples were immediately frozen at –80°C, lyophilized, and stored at –80°C until analyzed for MAAs.

Whole eyes were removed from freshly killed mice by dissecting away the ocular muscles, optic nerve, and connective tissues. A portion of the dorsal skin was

removed and the dermal side scraped with a razor blade to remove fat and connective tissue. Samples of liver were taken from the left lateral lobe and exsanguinated with isotonic saline. Portions of the proximal, middle and distal third of the small intestine, and of the large intestine, were removed and opened lengthwise. The mucosal surface of the intestines was rubbed gently with a blunt probe and thoroughly washed with isotonic saline to ensure that any gut contents containing MAAs were removed prior to tissue analysis.

### 2.4. MAA analysis

Lyophilized tissues and samples of the experimental diets were thrice extracted for 1 h in sufficient volumes of 80% aqueous HPLC grade methanol to cover the entire sample. Samples were sonicated briefly, on ice, during the second extraction. The pooled methanolic extracts were then centrifuged for 10 min to sediment any remaining cellular debris. Supernatants from the final spin were passed through a C-18 Sep-Pak Plus cartridge (Waters Corp., Milford, MA) to remove lipids and other chromatographically intractable materials. The ultraviolet/visible spectrum (200–750 nm) of the extracts was recorded on a Hitachi model U-3210 spectrophotometer (Hitachi Instruments, Inc., Tokyo, Japan).

High-performance liquid chromatography (HPLC) was used to identify and quantify the MAAs in the cleaned extracts. Optimum separation was achieved on our system using a mobile phase of 0.1% acetic acid in 55% aqueous methanol delivered over a Phenosphere C-8 column (4.6 mm ID × 250 mm) (Phenomenex, Torrance, CA) protected by a Brownlee guard column (Spheri-5 RP-8, 4.6 mm ID × 30 mm) (Applied Biosystems, Foster City, CA) at a rate of 0.8 ml min<sup>-1</sup>. Column eluate was monitored at 334 and 310 nm with a Waters model 490E UV detector. Peak areas were analyzed using a Waters model 746 integrator (Waters). MAAs were identified based on retention times and peak area ratios at the two wavelengths and confirmed by co-chromatography with authentic standards prepared by W.C. Dunlap. Extraction efficiency of the MAAs from tissue samples was 100%, as determined using the method in Ref. [14].

### 2.5. Removal of shinorine from an experimental meal

Four naive hairless mice were randomly selected and individually housed. Each mouse was fed a known amount of an experimental meal (≈ 8 g) that included 5% (w/w) freeze-dried *M. stellatus*. The concentration of shinorine in the experimental meals was determined as described above. Feces were collected twice daily from the onset of feeding and stored at –20°C. Following complete ingestion of the experimental meal, the

mice were again fed the standard laboratory diet to remove any shinorine remaining in the contents of the digestive tract. Feces were collected for an additional 3 days. The pooled feces were lyophilized and analyzed for the presence of shinorine as described above. Percent removal of shinorine during passage through the digestive tract was calculated as:

$$\% \text{ removal} = \left[ 1 - \frac{\text{shinorine in feces (nmol)}}{\text{shinorine in meal (nmol)}} \right] \times 100$$

## 2.6. Experimental medaka

Retired breeder medakas were a gift of Margaret Toussaint of the US Army Biomedical Research and Development Laboratory, Fort Detrick, MD. On arrival in our laboratory, approximately 150 medaka were housed in one 50-l tank for a period of 2 weeks. During this time they were fed twice daily to slight excess of a purified casein (PC)-based diet, modified from [11]. The tank was cleaned weekly to remove waste and discourage algal growth. During the 2-week period, four medaka were randomly selected and the tissues of their eyes and skin (tail fin) were analyzed for their initial complement of MAAs.

After 2 weeks, medaka were randomly assigned to one of two 50-l aquaria and to one of two experimental diets. One treatment group was maintained on a PC-based diet while the second treatment group was maintained on a PC-based diet with the addition of 10% (w/w) freeze-dried *Mastocarpus stellatus*. All other conditions in the two tanks (water chemistry, temperature (24°C), and rate of inflow) were monitored and maintained identically. The tanks were cleaned weekly throughout the feeding experiment. The room was monitored for ambient ultraviolet radiation as described above. There was no detectable UV emitted by the lights in the room.

## 2.7. Preparation of experimental diets for medaka

Dry ingredients were thoroughly mixed by hand, and oils were mixed with *tert*-butylhydroquinone and added to the mixture [11]. Distilled water was then added to the foregoing to form a slightly cohesive mixture. The final mixture was then passed through a # 14 stainless steel sieve to create uniform-sized feeding pellets of appropriate size for the medaka. The diet was then lyophilized and stored at –20°C until fed to the fishes.

The diet including 10% *M. stellatus* (double that used for mice) was prepared by adding 50 g of lyophilized, ground *M. stellatus* to 450 g of the dry ingredients and mixed well to ensure uniformity. The diet was then constructed as described above. The PC-based diets were analyzed for MAAs as described above.

## 2.8. Preparation of medaka tissue samples

At 38, 84, and 150 days following the onset of feeding of the experimental diets, four fishes were randomly selected from each of the two experimental treatment groups and analyzed for the presence of MAAs in the eyes and tail fin. Subjects were immersed in icewater and killed by decapitation. The eyes and tail fin were removed from each fish, immediately frozen at –80°C, lyophilized, and stored at –80°C until analyzed for MAAs.

## 2.9. MAA analysis

The procedure for extracting, separating, identifying, and quantifying MAAs was the same as described for the mouse diet and tissue samples, with the following modifications. Owing to the low volume of methanol used for the extraction of MAAs, it was not possible to sonicate the medaka tissues without rapid evaporation of the methanol. Therefore, this step was eliminated. Pooled extracts were not passed through a C-18 Sep-Pak Plus cartridge, again because of the low volume of methanol used. Rather, pooled extracts were spun at high speeds in a microcentrifuge. Extraction efficiency of the MAAs from tissue samples containing them was 100%, as determined using the method in Ref. [14].

## 2.10. Uptake of shinorine by human skin carcinoma cells

Human skin carcinoma (A431) cells (10<sup>6</sup> cells/60-mm dish) were provided by Rebecca VanBeneden, University of Maine. Cells were grown in Dubelcco's modified Eagle medium with 10% fetal bovine serum and L-glutamine (0.29 mg ml<sup>-1</sup>). All cell culture materials were from Gibco. After 24 h, aliquots of aqueous extract of *M. stellatus* (see below) were added to the medium and cells were grown for an additional 48 h in the presence of varying concentrations of shinorine. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay, which measures reduction of tetrazolium by mitochondrial dehydrogenases, was used as an indicator of cell viability [33]. To each sample well was added 0.5 ml of MTT (2 mmol ml<sup>-1</sup>); samples were incubated at 37°C for 1 h, after which they were washed three times with Hank's balanced salt solution (HBSS) followed by the addition of 1 ml of acid-isopropanol (100 μl 1 N HCl in isopropanol). Samples were mixed with a Pasteur pipet to dissolve the blue crystals and the solution was scanned from 280–620 nm; the absorbance at 570 nm was used for the analysis.

Viable extract-exposed cells were washed three times with HBSS buffer, trypsinized, scraped, and centrifuged at 225 × g for 4 min. The pellet was washed once more

with HBSS buffer and stored at  $-70^{\circ}\text{C}$ . Cells were thawed and extracted three times with  $100\ \mu\text{l}$  methanol. Extracts were combined and their shinorine content determined by HPLC as described for medaka tissues.

### 2.11. Preparation of extract of *Mastocarpus stellatus*

Freshly collected *Mastocarpus stellatus* was frozen in liquid nitrogen, crushed coarsely, then lyophilized. Approximately 30 ml of lyophilized material was covered with deionized water. After 1 h the mixture was centrifuged for 5 min, and 20 ml of the red supernatant was mixed with 200 mg activated charcoal and heated for 5 min in a boiling water bath. The mixture was centrifuged and the colorless supernatant filtered through a  $1\text{-}\mu\text{m}$  Super 200 membrane filter (Gelman). The extract was then sterilized by filtration through a  $0.22\text{-}\mu\text{m}$  filter (Gelman P/N 4523). The final shinorine concentration in this extract was determined by HPLC as described above.

### 2.12. Statistics

Mean concentrations of MAAs in the eyes and tail fin of medaka fed either of the two experimental diets were analyzed for statistical difference using a one-tailed Student's *t*-test (StatView II, Abacus Concepts, Inc., Berkeley, CA). Treatment means were considered significantly different at  $\alpha = 0.05$ . Concentrations of MAAs in the eyes and tail fin of medaka were related using correlation analysis and Dixon's test for suspected outliers [37]. Concentrations of MAAs in the four segments of the mouse gut were analyzed for statistical significance with a two-way analysis of variance (ANOVA), blocked by sampling period (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA). Possible differences among intestinal segments in shinorine concentration were evaluated for significance using a Student–Newman–Keul test.

## 3. Results

### 3.1. MAA complement in the experimental diets of SKH-1 mice and medaka

The methanolic extract from the Agway Prolab 3000 diet did not contain MAAs (Fig. 2A). The same diet including *M. stellatus* absorbed strongly at  $328\ \text{nm}$ , and HPLC analysis of this extract confirmed the presence of the MAA shinorine ( $0.554 \pm 0.062\ \text{nmol mg}^{-1}$  dry weight), and trace amounts of palythine and asterina-330 (Fig. 2B). Palythine and asterina-330 are not seen in this chromatogram, although they were present in

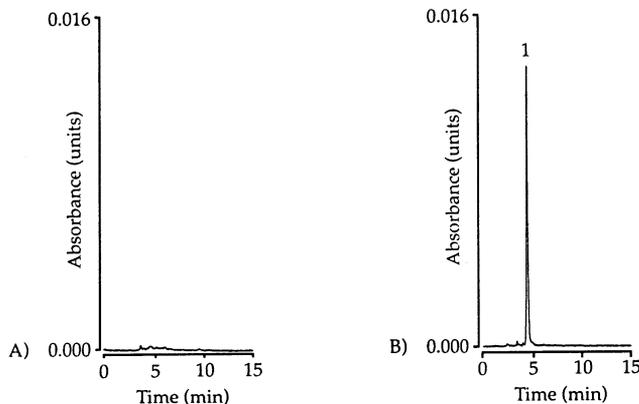


Fig. 2. Representative HPLC chromatograms (detection  $\lambda = 334\ \text{nm}$ ) of methanolic extracts of the Prolab 3000 diet (A) or the same diet with the addition of 5% (w/w) freeze-dried *Mastocarpus stellatus* (B). The lyophilized diet samples were thrice extracted in 80% HPLC-grade methanol and the pooled extracts diluted 1:10 for the chromatography. Both diet samples weighed 143 mg. 1, shinorine.

concentrations below the detection threshold for our system. This was determined by observing their presence in methanolic extracts of raw *M. stellatus* used to formulate the diet (data not shown).

Fig. 3 shows representative HPLC chromatograms of methanolic extracts of the purified casein (PC)-based diet fed to the medaka fish throughout this study. The extract from the PC-based diet with the addition of 10% (w/w) freeze-dried *M. stellatus* contained principally the MAA shinorine ( $0.675 \pm 0.046\ \text{nmol mg}^{-1}$  dry weight), and trace amounts of the MAAs palythine and asterina-330, while the PC-based diet alone contained no detectable MAAs.

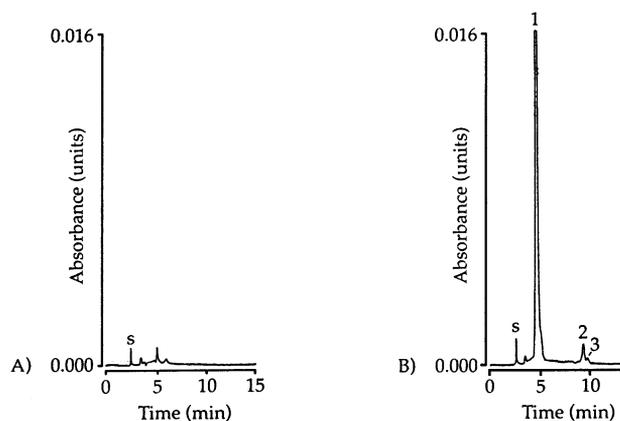


Fig. 3. Representative HPLC chromatograms (detection  $\lambda = 334\ \text{nm}$ ) of methanolic extracts of the PC-based diet for medaka (A) or the same diet with the addition of 10% (w/w) freeze-dried *Mastocarpus stellatus* (B). The lyophilized diet samples were thrice extracted in 80% HPLC-grade methanol and the pooled extracts diluted 1:2 for the chromatography. The diet samples were of similar dry weight, 270 and 271 mg, respectively. s, salts, 1, shinorine, 2, palythine, 3, asterina-330.

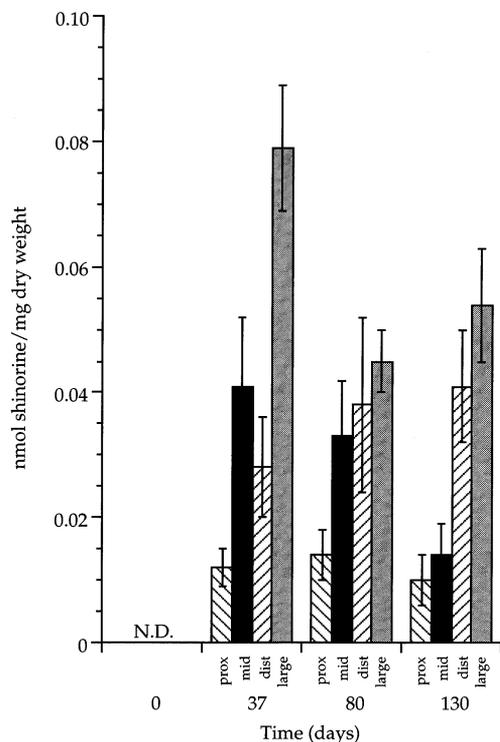


Fig. 4. Concentrations of shinorine in the proximal, middle and distal third of the small intestine, and the large intestine, of SKH-1 hairless mice maintained on a diet including 5% (w/w) freeze-dried *Mastocarpus stellatus* for up to 130 days. The tissues of the digestive tract were analyzed for the presence of MAAs on arrival in our laboratory and following maintenance for 37, 80, and 130 days on the experimental diets. The data are presented as mean  $\pm$  SEM,  $n = 4$ . The concentration of shinorine in the four intestinal segments varies significantly among segments (ANOVA,  $P = 0.0001$ ) but not temporally ( $P > 0.05$ ). N.D., no MAAs were detected in any tissue.

### 3.2. MAAs in SKH-1 hairless mice

No MAAs were detected in the tissues of the SKH-1 mice prior to the start of the experimental diets, or in the eyes, skin, or liver of mice at any sampling period through 130 days of feeding on either diet.

By 37 days of feeding on the *M. stellatus* diet, shinorine, the principal MAA in *M. stellatus*, was present in the tissues of the proximal, middle and distal thirds of the small intestine and those of the large intestine (Fig. 4). Representative HPLC chromatograms of methanolic extracts of the distal third of the small intestine of mice fed the control diet or the MAA-supplemented diet for 130 days are shown in Fig. 5. The unidentified peaks having retention times less than that of the shinorine peak increased continually in area down to a detection wavelength of 260 nm. Therefore, they are not MAAs, which have individual absorption maxima between 309 and 360 nm [15]. Co-chromatography with standards of mycosporine-glycine and mycosporine- $\alpha$ -taurine (MAAs having similar retention times to those of the unidentified peaks),

and shinorine, confirmed that the unidentified peaks are not these MAAs. The unidentified peaks appeared in all chromatograms of mouse tissues and did not vary in size between the two diet treatments. From day 37 until the end of this study (130 days), shinorine levels did not differ temporally within any of the four segments of the digestive tract (ANOVA,  $P > 0.05$ ). The concentration of shinorine differed significantly among the four segments of the intestines (ANOVA,  $P = 0.0001$ ), tending to increase in successive segments of the digestive tract (Fig. 4).

Palythine and asterina-330, known to be present in the *M. stellatus* used to formulate the mouse diet, were not detectable in the tissues of the digestive tract in mice fed the MAA-rich diet, most likely due to their deduced presence in only exceedingly small concentrations in the prepared diet (see above).

In naive mice fed a single experimental meal containing a known quantity of shinorine, all of the shinorine ( $100.5 \pm 5.8\%$ ) present in the meal was recovered in the feces.

### 3.3. MAAs in medaka

Palythine ( $0.016 \pm 0.005$  nmol  $\text{mg}^{-1}$  dry weight) and asterina-330 ( $0.001 \pm 0.001$  nmol  $\text{mg}^{-1}$  dry weight) were present in the eyes, but were not detected in the tail fin, of the medaka before initiation of the experimental diets. HPLC analysis of the flake fish food fed to the medaka prior to their arrival in our laboratory showed the presence of palythine, but not asterina-330 (data not shown).

Fig. 6 shows representative HPLC chromatograms of methanolic extracts of eyes of medaka maintained on the PC-based diet alone or the PC-based diet with added *M. stellatus*. Note that the principal MAA found

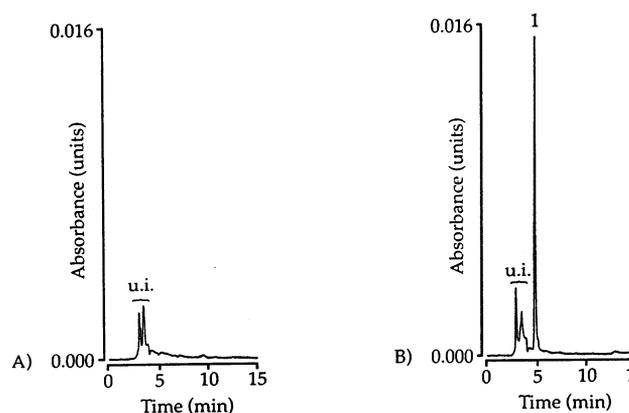


Fig. 5. Representative HPLC chromatograms (detection  $\lambda = 334$  nm) of methanolic extracts of the distal third of the small intestine of mice fed the ProLab 3000 diet (A) or the same diet with the addition of 5% (w/w) *Mastocarpus stellatus* (B) for 130 days. The tissue samples were of similar dry weight, 25 and 22 mg, respectively. The unidentified (u.i.) peaks are not MAAs (see Section 3). 1, shinorine.

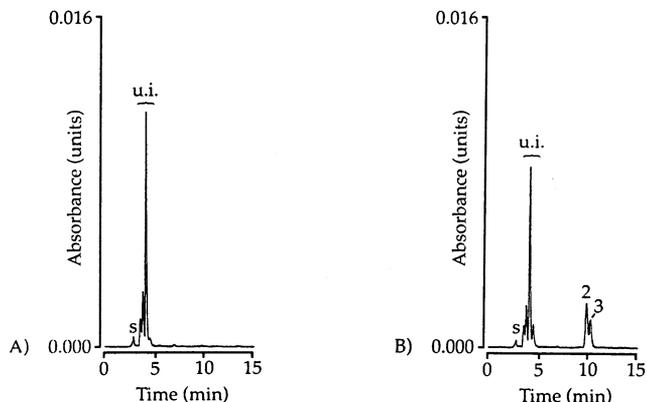


Fig. 6. Representative HPLC chromatograms (detection  $\lambda = 334$  nm) of methanolic extracts of the ocular tissues of medaka fish fed the PC-based diet (A) or the same diet with the addition of 10% (w/w) *Mastocarpus stellatus* (B) for 150 days. The tissue samples were of similar dry weight, 5 and 4 mg, respectively. The unidentified (u.i.) peaks are not MAAs (see Section 3). s, salts, 2, palythine, 3, asterina-330.

in *M. stellatus*, shinorine, was absent from the eyes of the medaka fed the diet including this alga, but that both palythine and asterina-330 (found only in trace amounts in *M. stellatus*) were present. The unknown peaks having retention times less than those of palythine and asterina-330 continually increased in area with decreasing wavelengths of detection and had a maximum absorption at 260 nm. Therefore, they are not MAAs (see above). These peaks were found in both the eyes and tail fins of medaka and did not vary in size between diet treatment groups.

The combined concentration of palythine and asterina-330 in the ocular tissues of the medaka fish increased during the feeding study from an initial value of  $0.017 \pm 0.005$  nmol  $\text{mg}^{-1}$  dry weight to a high of  $0.150 \pm 0.070$  nmol  $\text{mg}^{-1}$  dry weight after 150 days maintenance on the PC-based diet containing *M. stellatus* (Table 1; Fig. 7). Conversely, the concentration of these MAAs fell to undetectable levels in the eyes of medaka maintained on the unfortified PC-based diet for 150 days (Fig. 7). At each of the three sampling periods the concentration of the combined MAAs was significantly greater in the eyes of fishes fed the PC-based diet including *M. stellatus* than in the eyes of fishes fed the PC-based diet alone ( $P < 0.05$ ) (Fig. 7). The mean ratio of palythine to asterina-330 in the eyes of medaka at the three sampling periods (5.66:1) approximated that in the diet (6.36:1).

Fig. 8 shows the mean concentration of palythine and asterina-330 in the tail fins of medaka maintained on the two diets for up to 150 days. At each sampling period, palythine was present in the tail fins of the MAA-fed fishes and absent from the control fishes. The combined concentration of palythine and asterina-330 in the tail fin of medaka increased from undetected to

$0.006 \pm 0.003$  nmol  $\text{mg}^{-1}$  dry weight following 38 days of feeding on the PC-based diet including *M. stellatus*, significantly greater than control fishes maintained on the PC-based diet alone ( $P < 0.05$ ). The combined concentration of MAAs following 84 and 150 days of maintenance on the MAA-rich diet was not significantly greater than in control-fed fishes ( $P > 0.05$ ) owing to the high degree of variation in MAA content. Nevertheless, palythine was present in the tail fin of the MAA-fed fishes at all sampling periods up to 150 days and absent from the control fishes at the same sampling periods (Table 1).

The concentration of MAAs in the eyes of medaka was significantly correlated with the concentration of MAAs in the tail fins of the same fishes fed the MAA diet for up to 150 days (Fig. 9).

### 3.4. Uptake of shinorine by human skin carcinoma cells

The uptake of shinorine extracted from *M. stellatus* is concentration dependent and apparently saturable in viable A431 carcinoma cells (Fig. 10).

## 4. Discussion

The data reported here support the hypothesis that teleosts—in this case the Japanese medaka fish—can acquire MAAs from their diet. Two of the three MAAs present in the PC-based diet fortified with *M. stellatus* were also present in the ocular tissues of the medaka fed this diet, at significantly higher concentrations than in control-fed fish, at each sampling time (Fig. 7).

Medaka already contained MAAs in their eyes on arrival in our laboratory (Table 1). After maintenance for 150 days on the PC-based diet lacking MAAs, MAAs initially present in the eyes declined to undetectable levels. This further supports the supposition that MAAs are of exogenous origin in teleost fishes and may be depleted from tissues if unavailable in the diet (see also Fig. 7). Thus, medaka appear incapable of de novo synthesis of MAAs, nor do their enteric bacteria synthesize MAAs in detectable quantities.

To determine if the amount of MAAs in the eyes could be accounted for by that present in the food, we considered the total amounts of palythine and asterina-330 available to the fishes in their diet and calculated their potential assimilation by medaka. Using values in Ref. [9] for the daily ration,  $R$  (expressed as a percent of total body weight), of a similar size fish, and conservatively assuming  $\approx 80\%$  absorption efficiency for MAAs (cf. Ref. [7]), we calculated that in 38 days an average size medaka could have accumulated a total of 0.341 nmol of palythine and 0.054 nmol of asterina-330. Both values fall within the range of total mass of these MAAs in the eyes of medaka fed the PC-based diet

Table 1  
Concentrations of palythine and asterina-330 in the PC-based diet fortified with *Mastocarpus stellatus* and in the eyes and tail fins of medaka maintained on this diet for up to 150 days

Sample	Time (days)	[Palythine] (nmol mg <sup>-1</sup> dry wt.)	[Asterina-330] (nmol mg <sup>-1</sup> dry wt.)
Diet	—	0.018	0.003
Eyes	0	0.016 ± 0.005	0.001 ± 0.001
	38	0.086 ± 0.038	0.022 ± 0.011
	84	0.080 ± 0.028	0.017 ± 0.007
	150	0.134 ± 0.065 <sup>a</sup>	0.016 ± 0.005 <sup>a</sup>
Tail Fin	0	0	0
	38	0.006 ± 0.003	0
	84	0.011 ± 0.008	0.0003 ± 0.0001
	150	0.005 ± 0.003	0

Data are presented as mean ± SEM,  $n = 4$ .

<sup>a</sup>  $n = 3$ .

with added *M. stellatus* for 38 days (Table 1), taking into account the mean dry weight of the eyes (3.25 mg) at 38 days. Therefore, the amount of palythine and asterina-330 in the eyes of these fish can be accounted for by the amount of these MAAs supplied in their diet.

The ratio of palythine to asterina-330 was similar in the diet and in the ocular tissues of the fish, which is also consistent with a dietary origin of the compounds. Finally, the concentration of total MAAs (palythine + asterina-330) in the eyes of individual medaka was significantly correlated with the concentration of total MAAs in their tail fins (Fig. 9), which may indicate differences among these individuals in their ability to absorb and sequester MAAs. The levels of palythine and asterina-330 accumulated in the ocular tissues of

medaka are within the range of their concentration in the lenses of the 49 teleost species analyzed in Ref. [16], where these are among the predominant MAAs. Although these investigators reported MAA concentrations per mg protein in the lens and we report per mg dry weight in the entire eye, our values fall within their reported ranges. The principal MAA in *M. stellatus*, shinorine, was not present in either the tail fin or ocular tissues of the medaka at any of the sampling periods. This again is in accordance with the complement of MAAs reported in teleost lenses [16,39]: None of the lenses of the teleost species sampled in those studies contained shinorine.

Neither the physiological basis nor the adaptive significance of this seemingly preferential accumulation of certain MAAs is known. However, structural differences among these MAAs may account for this obser-

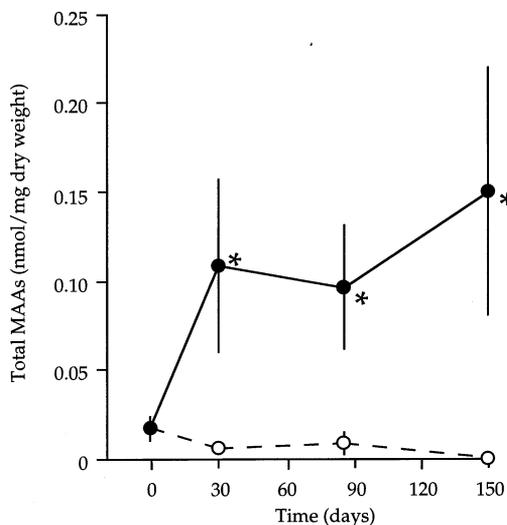


Fig. 7. Concentration of MAAs (palythine + asterina-330) in the eyes of medaka fed either the PC-based diet (○) or the PC-based diet with the addition of 10% (w/w) freeze-dried *Mastocarpus stellatus* (●). The eyes from medaka were analyzed for MAAs on arrival in our laboratory and following maintenance for 38, 84, and 150 days on the experimental diets. The data are presented as mean ± SEM,  $n = 4$  (except on day 150, where  $n = 3$ ). \*Statistically significant difference between treatment means ( $P < 0.05$ ).

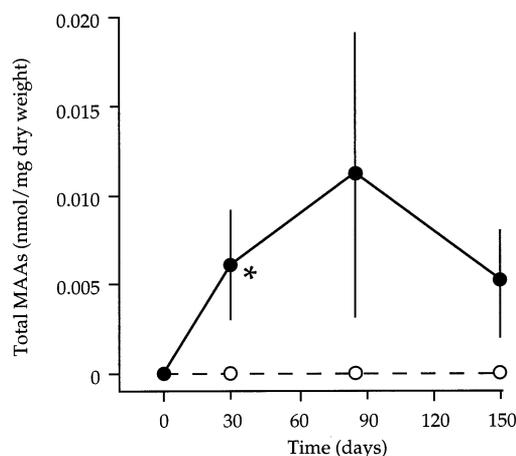


Fig. 8. Concentration of MAAs (palythine + asterina-330) in the tail fins of medaka fed either the PC-based diet (○) or the PC-based diet with the addition of 10% (w/w) freeze-dried *Mastocarpus stellatus* (●). The tail fins from medaka were analyzed for MAAs on arrival in our laboratory and following maintenance for 38, 84, and 150 days on the experimental diets. The data are presented as mean ± SEM,  $n = 4$ . \*Statistically significant difference between treatment means ( $P < 0.05$ ).

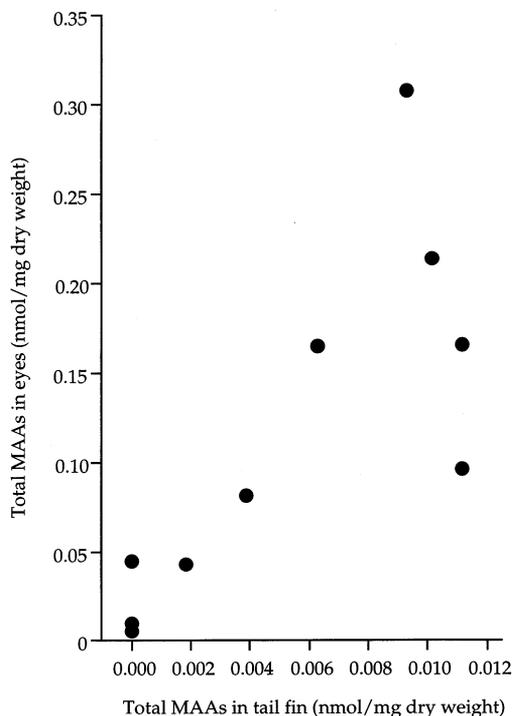


Fig. 9. The concentrations of MAAs (palythine + asterina-330) in the eyes and tail fin of medaka maintained on the PC-based diet including 10% (w/w) *Mastocarpus stellatus* for up to 150 days are significantly correlated ( $P = 0.008$ ,  $r^2 = 0.606$ ).

vation (see below). Feces collected several times each day from experimental medaka contained only trace amounts of dietary MAAs, and only one fecal sample among six tested contained quantifiable shinorine (data not shown). Whereas palythine and asterina-330 are retained in, especially, ocular tissues, the fate of ingested shinorine, by far the predominant MAA in the diet, is unknown. Shinorine might have been converted to asterina-330 within the fish in a manner analogous to that proposed for holothuroid echinoderms [15], thus obviating our calculation of the accumulation of asterina-330 from the diet (although no tissue nor fecal

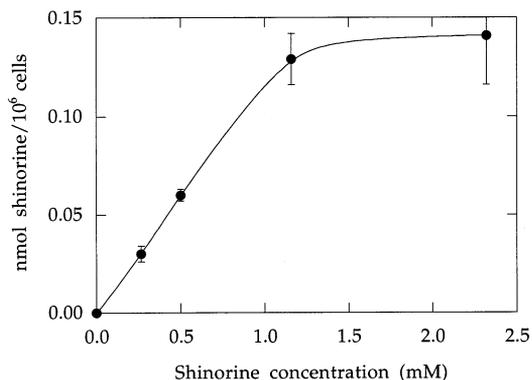


Fig. 10. Uptake of shinorine by human skin carcinoma (A431) cells from various concentrations in the culture medium during 48 h of exposure. Data are presented as mean  $\pm$  SEM,  $n = 3$ .

sample showed any trace of mycosporine–glycine, the proposed intermediate in the conversion of shinorine to asterina-330), or shinorine might have been catabolized. Alternatively, shinorine might have passed intact along the length of the gut and leached from the small fecal strands within minutes after defecation [26], too rapidly to be detected there.

The dietary accumulation of the MAAs palythine and asterina-330 in the ocular tissues of medaka and their absence from or presence in only trace amounts in the skin (Table 1) raises questions about the tissue specificity of various UVR-absorbing compounds and their importance to the fish. Deleterious effects of ultraviolet radiation in the skin of fishes are known [5,6]. One such effect, the nearly complete destruction of the epidermis of the plaice (*Pleuronectes platessa*) 48 h post-irradiation with environmentally relevant doses of UVR, demonstrates the destructive potential of UVR in fishes [5]. This may be of particular importance in fish aquaculture. In the wild, fish may be able to escape the harmful effects of UVR by modifying their behavior, e.g. swimming deeper in the water column or burrowing in the substratum. However, in fish-farming, the fish are grown at much shallower depths. The eggs, alevins, and fry of Chinook salmon, a popular aquacultured species, are particularly sensitive to UVR as indicated by increased mortality [12]. Thus, farm-raised fish may not be able to escape solar UVR unless they are housed in protective structures that attenuate environmental UVR, or allowed to occupy natural levels in the water column.

Other biochemical mechanisms may also allow fish to escape the harmful effects of solar UVR on the skin. Melanin concentrations in the skin are responsive to UVR exposure [31] and may effectively block or attenuate UVR there, and DNA repair mechanisms such as photolyase, known to exist in the epidermal and fin ray cells of medaka [18], may be of particular importance in this species. Whereas scales and skin may contain melanin or other opaque materials, the ocular lens must be transparent to permit vision, yet still be defended against solar UVR. Thus, it seems that medaka preferentially accumulate the MAA palythine and, to a lesser degree, asterina-330, in optically transparent ocular tissues.

Although the damaging effects of solar UVR on the skin of teleosts have been documented, little is known of such in their ocular tissues. Most of the work to date on fishes has been done using the lenses of an elasmobranch, the dogfish *Mustelus canis* [45–47]. These studies have shown that environmentally relevant near-UVR wavelengths can damage the cytoskeletal f-actin of the dogfish lens. Further, the authors have related this cytoskeletal damage to lens opacities, i.e. cataracts. Given the anatomical similarities between the eyes of elasmobranchs and teleosts [28], the eyes of

teleost fishes exposed to solar UVR may suffer similar damage. Reduction or loss of vision would most likely prove fatal in most fish species, as the procurement of food and the avoidance of predation would be seriously compromised. Thus, if UVR-absorbing MAAs in the ocular tissues do protect against the deleterious effects of solar UVR (an hypothesis which now requires testing), their presence in teleosts but not in elasmobranchs [16] may be of evolutionary significance.

Our experimental results, together with the known detrimental effects of solar UVR on fishes, also suggest that it may be beneficial to supplement the diet of aquacultured fish species with UVR-absorbing compounds. A ready source of one class of such compounds, the MAAs, are marine red algae (Rhodophyta).

Unlike fish, SKH-1 hairless mice seem incapable of fortifying superficial tissues (i.e. skin and eyes) with the MAAs provided in their diet. The absence of all of the dietary MAAs present in *Mastocarpus stellatus* from the eyes, skin, and liver of the mice, and the presence of shinorine only in the tissues of digestive tract, suggest that MAAs may be trapped in the space between enterocytes (some solutes are known to move through the tight junctions between adjacent enterocytes) [32,40], or transported across the enterocytic brush border and metabolized within these cells. The appearance in the feces of virtually all of the MAAs eaten by the mice argues against their transmural transport out of the intestine, or their large-scale metabolism there. Thus, the use of MAAs as an ingestible sunscreen in mammalian species does not seem efficacious.

The concentration of shinorine differed significantly among four segments of the mouse digestive tract, increasing in successive segments of the intestine, i.e. from the proximal third of the small intestine to the large intestine (Fig. 4). The trend toward higher concentrations of shinorine in more posterior portions of the gut might be explained by the progressive release of shinorine from the digesta, and by its concentration during its passage through the intestines as nutrients and water are removed, with small amounts of increasingly concentrated shinorine diffusing into the intercellular space. Regardless, the shinorine concentrations in the gut tissue are only  $\approx 10\%$  of that in the food.

What is the physiological basis for the taxonomic variation in dietary acquisition of UVR-absorbing MAAs? The principal region of MAA transport in the digestive tract of the green sea urchin may be the posterior gut because MAAs are most concentrated there [7]. That finding is consistent with other results [2,25] showing that the primary site of amino acid transport in sea urchins is the posterior gut, although it also mirrors our finding of higher shinorine concentrations in tissues of the large intestine despite the apparent lack of uptake of shinorine there. Although there is

a correlation between amino acid transport and MAA concentration in the posterior gut of sea urchins, and the process of nutrient absorption is similar in vertebrates and invertebrates [27,42], it is not known whether amino acid transporters in the urchin gut do in fact transport MAAs. Likewise, it is unknown whether amino acid transporters allow for the uptake of MAAs in fishes and whether the specificity of any such transporters differs in mice; if so, this would account for the observed differences between medaka and hairless mice in MAA accumulation.

It would seem, given the hydrophilic nature of MAAs, that some type of carrier is required to transport them across biological membranes, or that they pass into the bloodstream by paracellular absorption [27]. The acquisition of palythine and asterina-330, but not shinorine, from their diet by medaka is consistent with a complete absence of shinorine from the ocular tissues of taxonomically diverse marine teleosts in nature [16,39]. The observed selectivity of MAA bioaccumulation by medaka further suggests that MAA transport across the intestine is via specific or carrier-mediated mechanisms. In contrast to medaka, sea urchins are efficient at sequestering principally shinorine from their diet for accumulation in their ovaries and eggs [1,7]. These MAAs (Fig. 1) differ structurally and functionally in that palythine and asterina-330 (bonded to ammonia and ethanolamine, respectively, at the C1 iminocarbonyl position of the ring chromophore) are functionally neutral owing to zwitterion formation [20], whereas shinorine (bonded to an amino acid, serine, at the C1 position) has a net acidic character. These intrinsic differences might account for their differential absorption, perhaps by distinct carriers. However, if paracellular absorption (diffusion plus solvent drag) is responsible for the uptake of MAAs from the digesta, our results would imply a difference between fish and mice in the regulation of this absorptive mechanism. Critical, mechanistic studies on the uptake of MAAs must await the availability of radiolabeled compounds.

The possible accumulation of MAAs and their sequestration in certain organs is complicated by considering the results for hairless mice *in vivo* together with those for human skin cancer cells *in vitro*. Whereas the mouse apparently cannot translocate MAAs across its gut, human skin cancer cells can accumulate shinorine from the culture medium (Fig. 10). The apparent saturability of shinorine uptake by these cells is in keeping with a carrier-mediated transport mechanism, as might be expected for such a hydrophilic molecule. However, this conclusion is tempered by the long duration of the exposure (48 h), so the intracellular shinorine concentrations may also represent a steady state involving the uptake, release, and metabolism of this MAA. There is little reason to expect that these hypertriploid cancer

cells differ fundamentally from normal basal cells in their membrane transport characteristics, and their functional longevity owing to the overexpression of p53 allows use of this cell line [21] for kinetic and metabolic studies. The susceptibility of basal cells (which lack melanin) to solar UVR-induced carcinoma also was a factor in our choosing this cell line. We have not performed comparable experiments using cultured skin cells from mice.

Ingesting MAA-rich foods such as marine algae or MAA 'nutriceutical' supplementation to increase the protection against UVR in one's skin or ocular lenses is an appealing concept. However, our results indicate that this may not be feasible in mammals, although it might be useful in the aquaculture of fishes and invertebrates.

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